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PENETRATION OF THE INTESTINAL EPITHELIUM  
BY VARIOUS MICROORGANISMS

Akio Takeuchi

Walter Reed Army Institute of Research  
Washington, D. C.

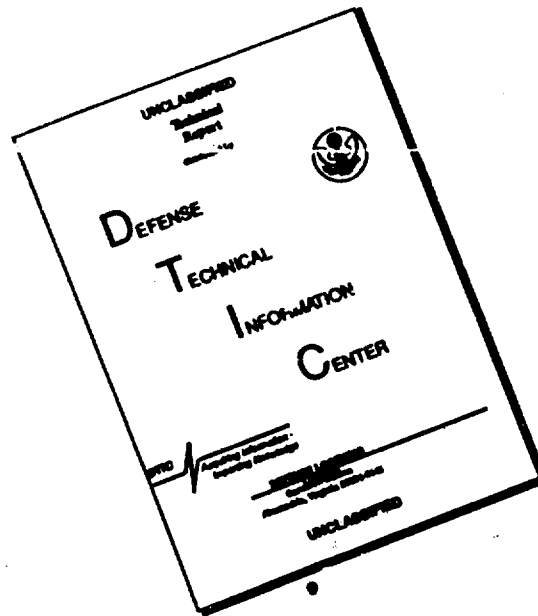
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PENETRATION OF THE INTESTINAL EPITHELIUM BY  
VARIOUS MICROORGANISMS (U)

AKIO TAKEUCHI, M.D.  
WALTER REED ARMY INSTITUTE OF RESEARCH  
WASHINGTON, D. C.

Many intestinal infections classed today as those of tropical diseases, such as dysentery and cholera are communicable diseases that were formerly found all over the world but have been eradicated by public health measure from temperate regions and from parts of the torrid zone. In the age of rapid transportation by air, the reintroduction of communicable diseases into areas once rid of them is distinctly possible. The recent appearances of cholera in Japan and smallpox in Europe is attributed to air travel. Moreover, enteric pathogenic bacteria once sensitive to antibiotics have acquired resistance to many antibiotics. For this reason, over the years our laboratory has studied pathogenesis of enteric infections of various types, with a modern knowledge and techniques including fluorescent antibody staining, histochemistry and electron microscopy.

In most enteric infections, penetration of the epithelial barrier of the gut by pathogenic microbes is the first step in establishing lesions in the host. Yet this first step had long been neglected. My study, over the past nine years at Walter Reed Army Institute of Research, has focused on the mechanism of this first step of infections and on the host-microbe interaction in the gut mucosa at the ultrastructural level, aimed at a better understanding of the pathogenesis of enteric infections.

Enteric microbes may be classified into four groups in accordance with their invasive potential into the host (16). The first group is characterized by a pathogen which readily invades through the epithelial barrier, multiplies in the mucosa and leads to a systemic invasion. *Salmonella* organisms belong to this group; they elicit a series of cytoplasmic alterations in the intestinal epithelial cells. The epithelial lining however, is far less damaged than that associated with a shigella infection (12,13). The second group is represented by shigella dysentery. Shigella organisms characteristically produce far more severe epithelial changes than salmonellae (11). Shigellae tend to reach and multiply in the epithelial lining;

Salmonella typhimurium (16) 1971  
Shigella flexneri (12,13)

15

TAKEUCHI

the organisms diminish in numbers from the luminal surface epithelium to the deeper portion of crypt cells. They are least frequent in the lamina propria, and are almost never disseminated by the blood stream (4). Members of the third group preferentially reside in the brush border and may or may not produce an overt acute inflammatory response and structural alterations of the gut mucosa. This group includes Cryptosporidium wrairi (21), and intestinal spirochetes (15, 16) attaches to the gut epithelium but causes severe symptoms thru absorption of their moiety. This group is exemplified by cholera vibrio (10, 20).

This paper summarizes my personal observations on the host-microbe interaction at the epithelial barrier in the first three types of enteric infections. For a discussion of cholera I refer to a paper by Sprinz (10).

The details of material and methods used in the following studies have been previously described in my original papers cited, and therefore will not be repeated in this paper.

#### 1. Penetration of the Ileal Epithelium by Salmonella

As far back as 1928 Ørskov and his colleagues fed mice with broth cultures of S. typhimurium and S. enteritidis and suspected that many bacteria passed through the intact intestinal epithelium (6). Florey in his histologic studies of S. typhimurium infection in guinea pigs, suggested that salmonellae passed through or between epithelial cells, possibly being transported by phagocytes (3). I restudied the problem in preconditioned guinea pigs which develop acute diffuse enteritis following peroral administration with  $10^8$  S. typhimurium (12,13). In the early stage of this infection, ample numbers of Salmonella uniformly penetrate the ileal epithelium which permits further clarification of Florey's concept. I shall follow the passage of salmonella organisms from the gut lumen into the small intestinal mucosa and shall describe the response of the cytoplasmic components of the epithelium to the penetration.

At 12 hours following peroral infections frozen sections treated with fluorescein labeled rabbit S. typhimurium antiserum show numerous organisms free in the intestinal lumen. Some of these are seen lining and in the lamina propria. Paraffin sections stained with Giemsa and 1-2u thick sections prepared by Epoxy resin stained with Methylene-blue-azure II reveal additionally that many bacteria are aggregated at the brush border and are also within the villus epithelium although absent in the crypt epithelium.

At ultrastructural level, the epithelial cytoplasm penetrated by organisms shows more vesicles than adjacent non-infected epithelial cells. Neutrophilic leukocytes between epithelial cells and in the lumen increase in number. Some neutrophils contain phagocytized bacteria. When bacteria are close to the brush border but more than 350 Å from the tip of the microvilli (critical proximity), the brush border is still unaltered (Fig. 1.1). As bacteria lie within

the critical proximity of the microvilli, first degeneration of the microvilli, then the apical cytoplasm begins; the filamentous core of microvilli and the terminal web topographically close to the bacterium become obscured. The microvilli begin to show such degeneration as elongation, fusion, swelling and budding Fig.(1.2). These alterations are absent in those portions of the brush border which are beyond the immediate vicinity of the bacteria. In fact, in these areas the microvilli, the filamentous cores and the terminal web are unchanged. The common portal of entry is through the brush border (Fig. 1.3A), but often is via the intercellular junctional complex between epithelial cells (Fig. 1.3B). More than one bacterium and occasionally a dozen may simultaneously penetrate one or two adjacent epithelial cells. As the bacterium moves further through the brush border (Fig. 1.3A), a cavity is formed before and around the advancing bacterium by a continuous degenerative process involving the microvilli and apical cytoplasm. This cavity is in open communication with the gut lumen (Fig. 2 and Fig. 1.4A). Degeneration of the apical cytoplasm is characterized by the following process (Fig. 2). A part of the host cytoplasm may bulge and project into the gut lumen or the cavity surrounding bacteria. These cytoplasmic projections appear as blebs distinct from the cytoplasm and presumably are pinched off and finally shed into a bacterium-containing cavity. Some blebs contain an aggregate of small vesicles which bear a striking similarity to a multivesicular body while most of the blebs lack such vesicles. As the bacterium advances further into the host cytoplasm, the bacterium-containing cavity which has previously communicated with the lumen is then transformed into the bacterium-containing vacuole which becomes separated from the luminal plasma membrane (Fig. 1.5A).

The entry of bacteria in the proximity of the intercellular junctional complex (Fig. 1.3B) also leads to the degeneration of adjacent microvilli and the corresponding apical cytoplasm and its terminal web. In the fashion illustrated in Fig. 1.3A, the cavity formed in the apical cytoplasm always displaces the intercellular junctional complex. Bacteria are capable of passing through the intercellular junctional complex including the tight junction (zonular occludens), intermediate junction (zonula adherens) or desmosome (macular adherens) (Fig. 1.4B). As the bacterium progresses, it is enclosed in a vacuole formed by the lateral plasma-lemma of two adjoining cells, together with degenerating microvilli and pinched-off cytoplasmic blebs or vesicles. The bacterium-containing vacuole appears to move laterally into the apical cytoplasm (Fig. 1.5B).

Thereafter, the bacterium-containing vacuole regardless of the portal of entry seems to take the same intracellular pathway and undergoes a similar transformation. In the supranuclear region, the bacterium-containing vacuole shrinks as its content, composed of blebs and vesicles is eventually replaced by acid-phosphatase rich electron-dense material (Fig. 1.6A). Otherwise the bacterium is surrounded by a discontinued membrane and dense material (Fig. 1.6B).

TABLE 1

Meanwhile, the structure of the overlying microvilli and of the apical cytoplasm including the terminal web is gradually reconstituted. Some bacteria in the supranuclear region are enclosed in the autophagic vacuole together with various host cytoplasmic components such as endoplasmic reticulum, mitochondria and ribosomes (Fig. 1.6C).

In the subnuclear region the bacterium is either surrounded by dense osmiophilic material (Fig. 1.7B), or enclosed by a single membrane (Fig. 1.7A). Thus far it has not been determined how the bacterium leave the cytoplasm of the intestinal epithelial cells and cross the basal plasmalemma into the lamina propria.

Twenty-four hours after infection the fluorescent antibody technique discloses that the number of free organisms in the gut lumen and of those in the epithelium has decreased markedly from that seen at the previous stage.

## 2. Penetration of the Colonic Epithelium by Shigella

Primates are the only animal species which are naturally subject to infection with dysentery bacilli. Commonly used laboratory animals such as rats and mice are naturally resistant to the infection with shigella bacilli. The resistance, however, is not absolute; guinea pigs can be made susceptible to orally administered shigella, provided they are preconditioned by starvation (4). Naturally occurring shigellosis in monkeys resembles shigella infection in man and can be produced experimentally by oral administration of shigella bacilli. For this reason, both preconditioned guinea pigs and monkeys have been extensively used in studies of shigellosis (5,22). This section outlines how virulent shigella bacilli invade the colonic mucosa of rhesus monkeys and how host cells respond to the invasion (14). The findings on the guinea pig shigellosis are included here if pertinent to the observations presented (11).

At 24 hours after peroral infection with Shigella flexneri 2a, the infected monkeys develop the clinical signs and symptoms of acute shigellosis. Frozen sections treated with fluorescent antibody disclosed moderate numbers of specifically fluorescing shigella organisms in the colonic lumen and rarely an occasional bacillus within epithelial cells. The number is so small and the distribution so irregular that any attempt to locate bacilli by electron microscopy is unsuccessful. Acute colonic lesions are well established 48 hours after infection. The colonic contents are scanty and consist of mucopurulent to mucohemorrhagic exudate covering the granular, unevenly swollen and edematous with patchy or diffuse hemorrhage. The small intestine appears normal but the ileocecal valve is edematous and occasionally hemorrhagic. Yet, gross ulceration is not observed throughout the colonic mucosa.

Histologically the colitis is characterized by a distinct gradation of the inflammation, diminishing from the luminal surface to the submucosa which shows but little reaction. The intensity of the inflammatory response corresponds to the degree and depth of bac-

terial penetration and parallels the damage to epithelial cells. The surface epithelium and the luminal aspect of the crypts are more severely affected than the deeper part of the crypts. The fluorescent antibody staining shows that specifically fluorescing shigella bacilli are identified predominantly in the surface epithelium and the more luminal crypt cells and are less frequently found in the deeper crypt cells. Least frequently they are in the lamina propria.

At the ultrastructural level bacteria in the epithelial cells are enclosed by a single or double membrane-limited vacuole, otherwise without such membrane enclosure they lie free in the cytoplasm or in the interepithelial space (Fig. 3). In the lamina propria, bacteria are always in neutrophils and macrophages where they are within membrane-bound phagosomes or lie free in the cytoplasm. Bacteria appear morphologically unaltered except for those in phagocytic cells which may show signs of degeneration.

The colonic epithelium shows a considerable spectrum of cellular alterations. Markedly damaged cells are seen between well preserved cells. Fat droplets measuring from 0.5 to 2  $\mu$  in diameter are present in surface and crypt epithelial cells irrespective of cellular alterations (Fig. 3). The luminal portions of the epithelium are in general attenuated. The PAS and Alcian blue-positive surface of the epithelium becomes thin or obscure corresponding to severe degeneration of microvilli including shortening and blunting of terminal web and the disappearance of the extraneous surface coats. In addition to the nonspecific alterations of the epithelial cells including changes of ER and Golgi, increase of autophagic vesicles and lysosomes, and the aforementioned accumulation of lipid droplets, there are increasing numbers of dark pyknotic epithelial cells. The severity of pyknosis appears to parallel the cellular degeneration processes. Extrusions from the epithelium occur with abnormally high frequency and not only from the villus surface but from the luminal aspects of crypts. Epithelial cells shed individually or in clusters or strips. The process of epithelial extrusion is altered; it is initiated by a detachment of a group of cells from the basal lamina while cells remain attached to each other at their lateral surfaces. The gap formed by this detachment is closed by a progressive movement of epithelial cells toward the luminal surface reflecting the innate tendency of the gut epithelium to cover the basement membrane. The final separation of the group of detached cells occurs at the desmosome. The epithelial cell extrusion contributes to the overall thinning of the epithelial lining. The formation of microulcers is characterized by a focal destruction of the basal lamina of the epithelium. As the defect widens, it is covered by fibrinous exudate or by leukocytes. It evolves into a superficial ulceration extending into the lamina propria. Pseudo-membrane lining the mucosal surface are infrequent. Emptying of goblet cells is pronounced.

The crypt epithelium shows increased numbers of mitoses and also undergoes the changes seen in the surface epithelium but to a lesser degree. However, the deeper portions of crypt cells are generally

less affected. Crypt distortion and abscesses are frequent. The lining epithelium is often flattened even with absence of neutrophils in the crypt lumen. Some leukocytes in the epithelial linings and in the crypt lumen show evidence of degranulation, however, most of them are well preserved.

### 3. Coccidia and Spirochetes on the Gut Epithelium

A. Coccidia-Cryptosporidium wrairii: Infestations of coccidia in the small intestine of guinea pigs which otherwise are free of enteric pathogens (21). Cryptosporidia infections do not produce overt clinical manifestations but are accompanied by chronic inflammatory responses in the mucosa. The coccidia are characteristically localized at the brush border of the small intestine (Fig. 4); they are not found in the large intestine nor do they invade beyond the brush border. In paraffin sections stained by Giemsa or H & E, the coccidia are found most often near the tip of villi, their presence being accompanied by chronic inflammation in the mucosa and alterations of villus architecture. The villi are short, broad, and often flattened.

In the ultrastructural studies, microvilli disappear, or are pushed aside at the site of attachment (Fig. 5). The organism is firmly attached to the luminal plasma membrane of the epithelial cell; the outer membrane of the organism is fused and becomes continuous with the luminal host membrane, while membranes develop into multiple infoldings at the parasite cytoplasm which is topographically above the attachment zone (21). In the host cytoplasm, cellular organelles near the attachment zone are generally displaced laterally. In the infected cells, mitochondria are swollen and the cisternae of ER are dilated.

B. Intestinal Spirochetes: Spirochetes have long been recognized in the gastrointestinal tract of various animal species including man. Yet, little is known about the significance and pathological potential of intestinal spirochetes. Some thought them enteric pathogens while others considered them commensal organisms and a part of the normal microbial flora. This discrepancy results mainly from the fact that most spirochetes are fastidious anaerobes and are difficult to culture. Our studies indicated that spirochetes occur in the brush border of the colonic epithelium of normal rhesus monkeys (12%) and also of normal human subjects (3%). In paraffin sections stained by H & E, the brush border of the normal colonic epithelium is rather narrow and is uniformly stained with eosin. On the other hand, the brush border region infected by spirochetes is broader than the normal counterpart and is basophilic. The spirochete-infested brush border is stained strongly by Giemsa and specifically colored by silver stain. Spirochetes are never seen deep in the crypt epithelium. There are no histologic changes in the spirochete-infected mucosa; the epithelial cells remain unaltered and the lamina propria lacks any inflammatory response.



At the ultrastructural level, spirochetes are easily recognized by their characteristic spiral shape. They are exclusively localized at the brush border region affecting large numbers of epithelial cells. Innumerable spirochetes frequently replace the microvilli with only occasional remnants remaining (Fig.6). The fuzzy coat of the glycocalyx largely disappears but a thin cap of fuzz is occasionally present over the apex of microvilli. The microvilli and terminal web for the most part are indiscernible, while the rest of the cytoplasmic components of columnar cells remain unchanged. These ultrastructural characteristics of spirochetes are indistinguishable from those of Borrelia vincentii, a common oral spirochete (Fig. 6,7).

We also investigated intestinal spirochetes by a new type of electron microscope, the scanning electron microscope. Utilizing great depth of focus and superior scanning capability, this new instrument provided the unique opportunity to visualize the three-dimensional view of spirochetes at their natural environment (18). Three-dimensional observations were made at magnifications 100-10,000. At 1000 magnifications, the normal surface is completely replaced by the granular surface representing multiple spirochetes at brush border (Fig. 8). At 10,000 magnification, individual spirochetes are well distinguished and are oriented perpendicular to the mucosal surface (Fig. 9). They are 5u long and 0.5u wide. As many as 1800 organisms are present per square millimeter.

#### COMMENTS:

The brush border of the small intestine is an important region from the physicochemical point of view. The surface of the microvilli is covered by a fuzzy extraneous layer composed of an acid mucopolysaccharide complex. The brush border region contains various phosphatases and is the site of the terminal hydrolysis of disaccharides and peptides prior to absorption. To what extent this micro-environment may provide a chemical barrier against microbial penetration is unclear. Electron microscopy demonstrates the impact of various organisms upon this physicochemically important portion of the intestinal mucosa. In particular it clarifies the mechanism of the initial penetration and passage of salmonella organisms from the gut lumen into the intestinal epithelium. Most frequently several pathogens simultaneously invade a single epithelial cell or several adjacent cells through the brush border. Organisms are also capable of penetrating the epithelial lining through the intercellular junctional complex. The approach of a single pathogen to within the critical distance from the brush border triggers a sudden local degeneration of the microvilli. On the other hand, mere presence of even large numbers of salmonellae, in the lumen beyond the point of critical distance appears to have no effect on the intestinal epithelium. After penetration, the impact of the organism upon the host cell is localized. The bulk of cytoplasmic components of the epithelial cells is well preserved despite the

enclosure of many organisms in membrane-bound vacuoles. Organisms not enclosed by a membrane seem also to produce a strictly local effect. These observations indicate that salmonellae in their passage thru the intestinal epithelial lining have only a limited cytotoxin action. The host cell is capable of confining the organisms and of preventing extensive cellular damage. In fact it is capable of transporting the pathogen thru and out of the cell at the same time it is repairing itself along the brush border.

The intraepithelial salmonella-containing vacuole as it descends from the apical to the deeper cytoplasm undergoes a reduction in size and a conversion of the vacuolar contents into acid phosphatase-rich material. The evolution of the organism-containing vacuole bears a close resemblance to the intracellular digestive process by acid phosphatase and nonspecific esterase in mammalian tissue culture cells (5). An analogous intracellular response and the mobilization of lysosomal enzymes probably occur in intestinal epithelium after penetration. All salmonella organisms within epithelial cells and most of those in the phagocytes are morphologically intact, an observation which appears to apply to shigella and intestinal spirochetes. Moreover, this signifies that in the epithelium, in spite of intracellular segregation by host cell membranes and digestion of host cytocomponents within the vacuoles by acid hydrolases, organisms have the capacity to survive and multiply.

During and after penetration of the gut epithelium by pathogens, epithelial cells desquamate excessively into the gut lumen. We have studied this phenomenon in both salmonella and shigella infections. Under normal conditions an epithelial cell produced in the crypt migrates along the villus and is extruded at the villus tip. Using radioactive thymidine, it has been demonstrated that the epithelial cell renewal is significantly shortened in salmonella enteritis (1). The epithelial cell in the acute mucosal lesion reaches the villus tip within 24 hours, while the normal epithelial cell arrives at the extrusion zone in about 48 hours. This indicates that crypt cells are stimulated to undergo mitosis at more frequent intervals in order to replenish the cell loss from the villus. Accelerated and excessive cell extrusions occur not only at the normal site, but also at such abnormal sites as the mid-villus. The increased cell turnover and extrusion greatly reduce the total number of mature cells and bring immature cells to the luminal surface. This abnormal cell desquamation is a common event in the gut mucosa under various inflammatory conditions including acute bacterial infections, Entameba histolytica infection in guinea pigs (17) and non-tropical sprue in humans (2). Thus, this phenomenon represents one of the general responses of the gut mucosa to injury.

Shigella organisms have a unique position among enteric bacterial pathogens in that they preferentially reside in gut epithelium. Dysentery bacilli can divide in this specific microenvironment and presumably have the ability to spread through the lateral plasma membrane from one epithelial cell to the next in addition to their capacity to penetrate the brush border. While I have only indirect

evidence of a lateral transfer of bacilli, this view is confirmed by a cinematographic study of shigella-infected HeLa cells; bacilli are observed not only to penetrate, but once inside the non-motile organism becomes highly motile and is seen to invade adjacent tissue culture cells through the lateral plasmalemma (18).

When preconditioned guinea pigs are challenged orally with virulent and avirulent strains derived from the same parent strain of *Shigella*, only the virulent strain invades the mucosal barrier and multiplies in the mucosa, while the avirulent strain is unable to penetrate the epithelial lining (4). From this it appears that strain differences in virulence play a decisive role in determining invasive capacity. We believe that a confrontation between potentially invasive enteric pathogens and the physicochemical barrier of the brush border is successfully overcome only by virulent organisms, which is then followed by epithelial penetration and multiplication in the host.

The close affinity of organisms to the brush border of the gut epithelium is characteristic for cryptosporidia and intestinal spirochete infections. A similar relationship has been recognized in infestations with *Streptobacillus moniliformis* and *Giardia lamblia* in man (7) and *Giardia muris* in mice (19). In giardiasis the sucker plate covers the epithelial cell and is often implanted in the extraneous coat of microvilli (7,19). In cryptosporidiasis the luminal plasmalemma of epithelium is intact albeit distorted, while the plasma membrane of the organism develops into a specific attachment device by which the parasite is secured at the brush border (21). In spirochete infections, such an attachment device is absent, the luminal plasma membrane of epithelial cells being simply in contact with the cell wall of the organisms.

The scanning electron microscope has shown for the first time how intestinal spirochetes inhabit their natural environment; three-dimensional views reveal that organisms flourish almost as a pure culture on the colonic mucosa. At the same time this new tool clearly demonstrates the spatial relationship between spirochetes and the mucosa. No organisms are randomly oriented on the mucosa; all are well-oriented perpendicular to the surface. This relationship suggests that organisms may utilize their forward portion for nutritional intake from host cells. It is also possible that this unique orientation permits equal access to host cells for each organism while simultaneously maximizing the number of spirochetes with such access.

#### CONCLUSION:

While enteric infections share a common basic pattern of morphologic responses in the host, the nature of microbes and the types of host-microbe interactions result in significant differences in pathogenic mechanisms. Electron microscopic observations have greatly contributed to the clarification of these mechanisms.

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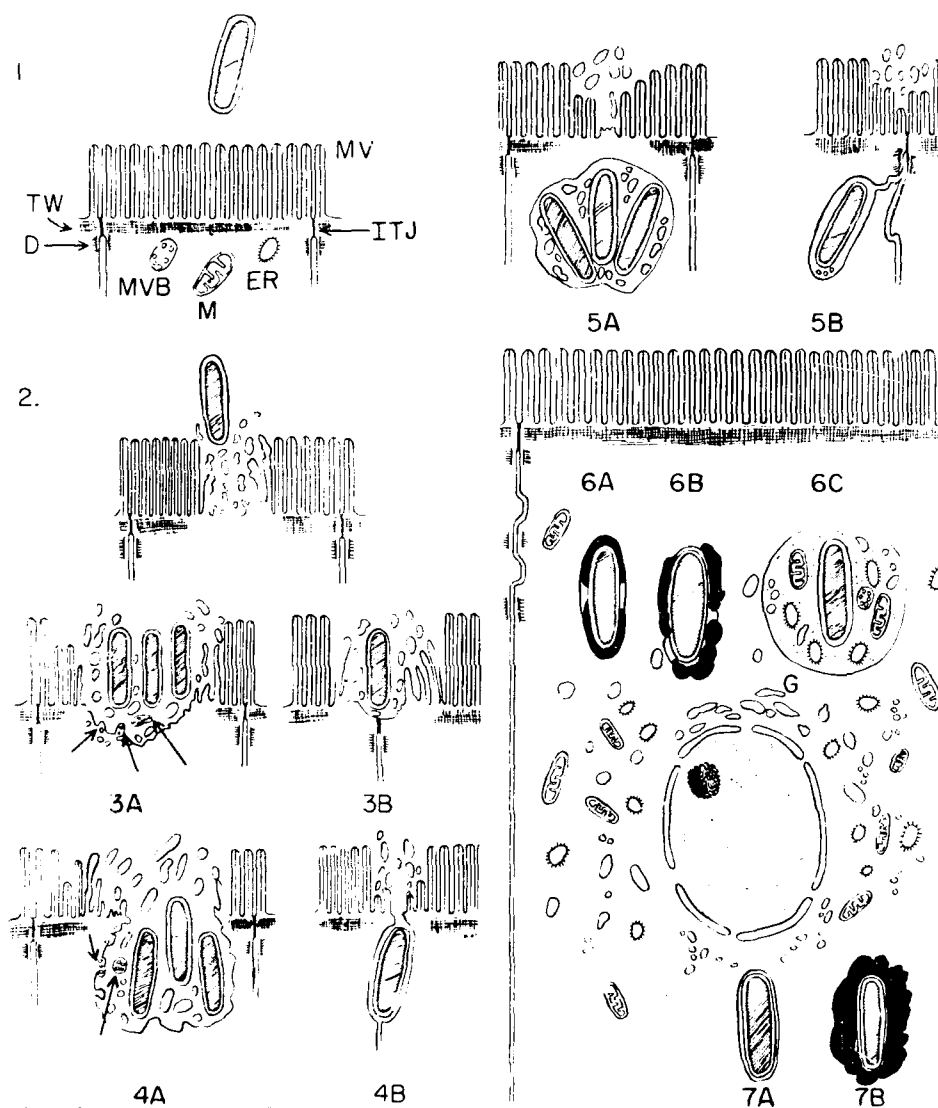


Fig. 1. A schema illustrates the process of penetration (1 thru 7) of *S. typhimurium* into the small intestinal epithelium.

The brush border consists of microvilli (MV) and terminal web (TW) ending at terminal bar between intercellular junctional complex (ITJ) and desmosome (D). Mitochondria (M), endoplasmic reticulum (ER) and multivesicular body (MVB) are present below the terminal web. The process of penetration (1 thru 7) in detail refers to the text.



Fig. 2. The microvilli, terminal web and apical cytoplasm are replaced by a cavity in which bacteria together with degenerating microvilli, blebs and vesicles are present. Bleb-like projections with (B) or without small vesicles (A) arise from the host cytoplasm and are pinched off (C) into a cavity in which degenerating microvilli are also present. There are increasing numbers of small vesicles in the host cytoplasm. An intercellular junctional complex is laterally displaced (arrows). G Golgi. Corresponds to Fig. 1-4A. X27,000.



Fig. 3. Luminal aspect of colonic epithelial cells at crypt showing penetrated secondary villi. Two bacteria indent the lateral plasma membrane and appear to push into adjacent cells (dotted line). This may represent a process of lateral spread of the organisms. One bacterium shows fission (arrow) and lacks membrane infoldings. Microvilli and corresponding web are absent. The lumens of the Golgi (Go) and occasional ER are dilated. Fibroblasts through ER are decreased in number. F fat droplets. X 5,000.

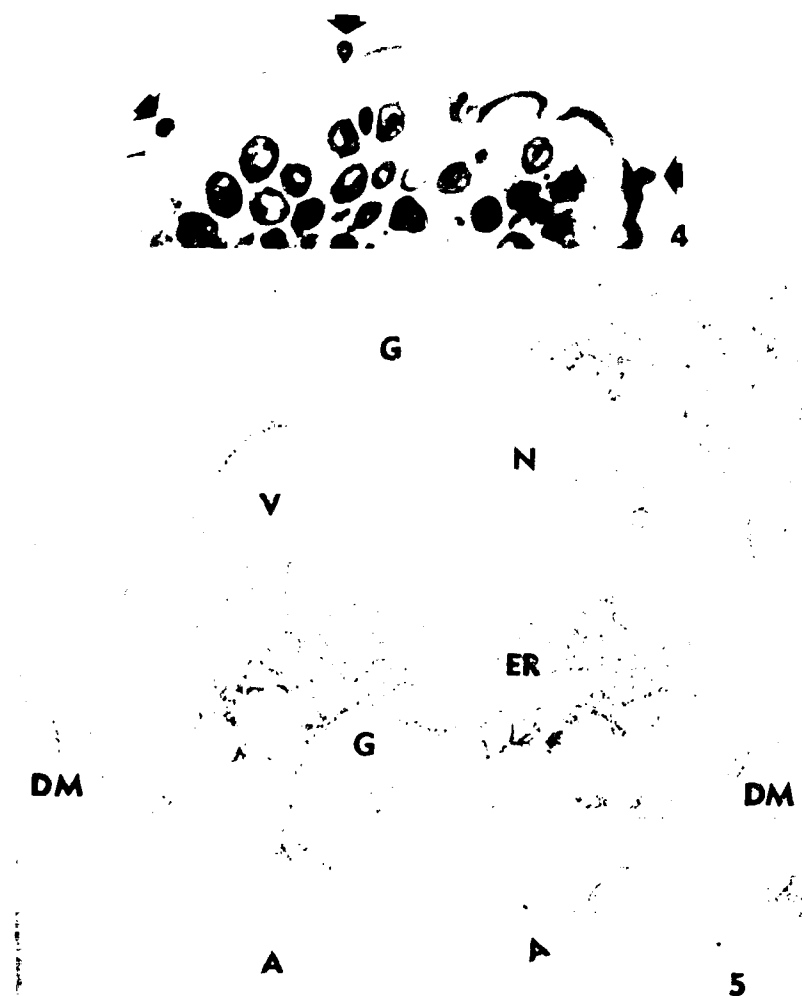


Fig. 4. Ileal mucosa at mid-villus showing cryptosporidia at the brush border. The brush border is intact except for the site of implanted coccidia (arrows). X 400.

Fig. 5. Ileal epithelial cells at mid-villus, guinea pig showing cryptosporidia at the brush border. A macrogametate is embedded in the brush border region in which the terminal web is indistinct. The organism is secured in the host cell at the base, where the cell wall of the organisms is fused with the plasmalemma. Microvilli at the attachment (A) disappear but distant microvilli (DM) are intact. N: nucleus G: granule. ER: endoplasmic reticulum. V: vacuole X17,000.





Fig. 6. Spirochete-infected brush border of the colonic epithelium. Multiple spirochetes replace microvilli with occasional remnants remaining. The cell wall of spirochetes closely contacts with the invaginated host plasmalemma. Organisms exhibit axial fibers, characteristic for spirochetes, appear to be wound around the cytoplasm. X62,500.

Fig. 7. Cross section cut at the level of the dotted line in Fig. 6. The fibers are situated between the cell wall and the cytoplasmic membrane. Between spirochetes are cross section of microvilli. X74,000. Axial fibers (parenthesis), cell wall (large arrow) cytoplasmic membrane (small arrow).

Fig. 8. Scanning electronmicroscope illustrates spirochete-infested colonic mucosa. The normal mucosa surface is completely replaced by numerous spirochetes. The central hole is a crypt opening. X3,000.

Fig. 9. Magnification from the square of Fig. 8 illustrates the individual spirochetes. X10,000.